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The Role of Social Environment and Genomic Plasticity in the Maintenance of Alternative Mating Strategies in Sailfin Mollies

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THE ROLE OF SOCIAL ENVIRONMENT AND GENOMIC PLASTICITY IN THE MAINTENANCE OF ALTERNATIVE MATING STRATEGIES IN SAILFIN MOLLIES

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by
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ABSTRACT

Sailfin molly fish, *Poecilia latipinna*, members of the family Poeciliidae, show extensive polymorphism in male body size, degree of ornamentation and mating behavior repertoires. They are a striking example of a species with alternative male mating strategies that result from an association between life history trait variation and developmental plasticity of mating behaviors. Sailfin molly male body size correlates to their lifelong mating strategy and male size at maturity is fixed and shows a continuous range of male sizes in natural populations. In this species, some phenotypes have 'fixed' mating strategies (either sneaking copulations by small males or courting females by large males) while intermediate-sized males respond plastically to their social environment and switch their mating strategy from either courting or sneaking behavior depending on the social context of male competitors in their environment. Using RNAseq, we profiled differential gene expression between the brains of males with the fixed mating strategies (small versus large males), and among social rearing conditions (presence of females or males of small or large size) in males with the more plastic mating strategy (intermediate males). Larger genomic responses were discovered between males with the alternative fixed mating strategies than among males with the plastic strategy. We found that genes relevant in memory and associated processes were significantly small male-biased more often than by chance, while genes involved in protein synthesis and immunity were up-regulated in large males significantly more often. We also found that social condition during ontogeny does not appear to strongly influence differential gene expression between small and large size classes of males with

fixed mating strategies, but social rearing environment does influence gene expression patterns to some degree in intermediate males, particularly in response to juvenile development with a social environment containing small males. Our results revealed that fixed mating strategies by large and small male sailfin mollies have broad neurogenomic responses due to alternative mating strategy use, while more plastic mating strategies of intermediate males are more influenced by the social conditions these males experience during ontogeny.

DEDICATION

I would like to dedicate this thesis to my husband, Mario. His unwavering support and belief in me made all of my graduate work possible.

ACKNOWLEDGMENTS

I would like to thank the incredible team of people that helped produce this thesis. I would especially like to thank my co-advisors, Margaret Ptacek and Christina Wells. Thank you, Margaret, for pushing me to be not only a better scientist, but also a better person. I feel very lucky to have been your graduate student. Christina, thank you for believing in my abilities and reminding me to approach biology with wonderment, intuition and confidence. I would also like to thank my other committee members, Matt Turnbull and Amy Lawton-Rauh. Matt, thank you for teaching me to practice science ethically and rigorously, while still maintaining kindness and compassionate mentorship. Amy, thank you for sharing your joy in genetics, and teaching me the importance of outreach and education.

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CHAPTER ONE

UNDERSTANDING THE ROLE OF PHENOTYPIC PLASTICITY IN THE PRODUCTION OF ALTERNATIVE REPRODUCTIVE STRATEGIES

INTRODUCTION

Variation among individuals within a species is essential for natural or sexual selection to lead to the evolution of adaptive phenotypes. Yet, paradoxically, strong selection often leads to the decrease in variability of traits among individuals when selection results in a single phenotype reaching a selective optimum (Lewontin 1974; Charlesworth & Hughes 2000; Mitchell-Olds *et al.* 2007). The maintenance of polymorphism in phenotypes has been of general interest to evolutionists and in particular, the role that phenotypic plasticity may play in generating phenotypic variability as one solution to this evolutionary paradox. Phenotypic plasticity, the ability of a single genotype to produce alternative phenotypes in response to environmental variation, allows for both the origin and maintenance of polymorphic traits (West-Eberhard 2003; Auld, Agrawal & Relyea 2009; Scheiner & Holt 2012). Variable environmental conditions and underlying plasticity genotypes can allow for evolution of new specializations within the novel trait without elimination of an established trait and lead to the maintenance of variability in mating strategies within species (West-Eberhard 2003). Plasticity in condition-dependent reproductive traits, has led to the expression of alternative reproductive strategies or tactics (ARTs) in a number of animal species (Gross 1996; Brockmann 2001). While numerous studies have examined how ARTs are maintained through fitness differences in morphological, behavioral and physiological traits as a result of some form of balancing selection (e.g., negative frequency dependent natural or sexual selection), we still know

little about the underlying genetic basis of these plastic responses or how changes in genomic architecture can favor the evolution of adaptive plasticity in life history and reproductive traits (Wimberger 1992, Rousseau & Dufour 2007).

Teleost fishes provide some of the most well-known examples of polymorphic reproductive strategies, where individual males often adopt ARTs to maximize reproductive success when males must compete for mating access to females within the constraints of genotypic, social, and ecological conditions (Gross, 1996; Neff & Svensson, 2013). In taxa that engage in ARTs, there are at least two male morphs (Taborsky *et al.* 2008). Type 1, bourgeois males, fully express male secondary sexual characters and compete for the monopolization of access to females along with expressing typical male behavior such as aggression, territory or nest defense and courtship (Taborsky *et al.* 2008). Type II males, parasitic or sneaker males, do not express male secondary sexual characters or behavior and typically sneak fertilizations (Taborsky *et al.* 2008). Interestingly, the mechanisms maintaining ARTs differ significantly across fish species and individuals. In some individuals, tactics are fixed and can occur either through inherited genetic polymorphisms (Lank *et al.* 1995; Schuster & Sassaman 1997; Kupper *et al.* 2016; Lamichhany *et al.* 2016), conditional-dependent switches triggered before sexual maturation (Gross 1996; Taborsky 1998; Gross & Repka 1998) or a combination of genetic and environmental factors (Piche, Hutchings & Blanchard 2008; Neff & Svensson 2013). In other individuals, tactics are plastic throughout their reproductive life, in response to social or abiotic environmental cues (Gross 1996; Moore 1991; Taborsky, Oliveira & Brockmann 2008). However, behavioral

plasticity appears to carry significant physiological costs that can favor the fixation of alternative male phenotypes (Fraser *et al.* 2014). Recent advances in sequencing technology allow for exploration of how variation in gene expression contributes to behavioral and morphological variation among different alternative phenotypes.

Understanding how reproductive plasticity arises and is subsequently maintained genetically is crucial to understanding evolutionary phases leading to speciation, as the maintenance of polymorphism in mating strategies can influence how quickly populations can diverge (Coyne & Orr 1998). When novel alternative phenotypes arise within a population they can go to fixation leading to rapid population divergence or be maintained as polymorphisms, slowing the rate of divergence. Prior studies have found that gene expression differences among discrete alternative male phenotypes reflect the behavioral and morphological differences observed (Aubin-Horth *et al.* 2005a; Fraser *et al.* 2014; Schunter *et al.* 2014). Growth rate and length of juvenile growth period are known to be the primary causes of the development of alternative male phenotypes within species (Alonzo *et al.* 2000). Variation in the expression of growth related genes reflects the differential needs for growth and development between alternative male phenotypes (Aubin-Horth *et al.* 2005a; Fraser *et al.* 2014). Social influences can also lead to baseline alternations in brain gene expression between alternative male phenotypes (Aubin-Horth 2005b; Fraser *et al.* 2014). Indeed, changes in social environment during ontogeny may favor developmental plasticity in reproductive strategies (Kasumovic & Brooks 2011). This 'socially-cued anticipatory plasticity' (SCAP) hypothesis proposes that developmentally plastic mating strategies evolve to allow individuals to produce

phenotypes well-adapted to the social environments they will encounter when mature (Kasumovic & Brooks 2011).

In sailfin mollies (*Poecilia latipinna*) populations have males that exhibit both fixed and plastic alternative mating phenotypes (males contribute only sperm to reproductive success). Male sailfin mollies vary widely in body size and size-associated mating behaviors, and compete with other males for internal fertilization attempts with females (Travis 1994; Farr, Travis & Trexler 1986; Trexler, Travis, & Trexler 1990). Males can exhibit different rates of either courtship or sneaking mating behavior (termed gonopodial thrusting), depending on the fixed male body size that is determined at the time of sexual maturity (Ptacek & Travis 1996; Seda, Childress & Ptacek 2012). Large males (> 44 mm) perform courtship displays by raising and spreading their large iridescent dorsal fins (sailfin) in front of the female while curving the body in a C-shape (Farr 1989). Small males (<30 mm) typically sneak copulations by thrusting their gonopodium (sperm transfer organ) at the female's gonopore (Ptacek & Travis 1996). A third male phenotype is intermediate in size (31-44 mm) and associated secondary sexual characteristics and is plastic in mating behavior depending on the social environment. Intermediate males exhibit higher rates of courting behaviors in social environments with no males or small sneaker males and higher rates of sneaking behavior in the presence of competitor males, particularly large males (Trexler & Travis 1990; Ptacek & Travis 1996; Fraser *et al.* 2014). The presence of both fixed and plastic mating behaviors among males in the same population makes *P. latipinna* a particularly useful model for

investigating genomic mechanisms underlying the role of plasticity in the maintenance of alternative mating strategies.

Numerous phylogenetic and behavioral studies have been conducted in *P. latipinna* examining within and between population variability in rates of mating behaviors associated with the alternative male phenotypes that are a result of the differences in age and size at maturity of males (Farr, Travis & Trexler 1986; Trexler *et al.* 1990, Trexler & Travis 1990; Ptacek & Travis 1996; Seda *et al.* 2012). However, far less is known about the underlying genes involved or how gene regulation among males of different sizes and mating strategies may differ. One study examining differential expression (DE) between small and intermediate males of *P. latipinna* did find that nearly a third of all brain transcripts differed in expression pattern (Fraser *et al.* 2014). In both Atlantic salmon (*Salmo salar*) and sailfin mollies, the sneaking strategy was associated with upregulation of genes involved in neurotransmission, learning and mechanosensory processes, suggesting that sneaking may be more cognitively demanding than courtship (Aubin-Horth *et al.* 2005a; Fraser *et al.* 2014). However, neither DE of genes at the time point of sexual maturity nor the influence of social environment during juvenile development on such DE has been investigated in *P. latipinna* to better understand how alternative mating strategies are developed during ontogeny.

In non-model species like *P. latipinna*, it is often difficult to study DE at the gene level in the absence of a quality reference genome (Scoville & Pfender 2010). Thus, for this study we used RNAseq to first characterize the brain transcriptome of the male sailfin molly, then to examine molecular differentiation among groups. Specifically, we

aimed to (1) investigate differential gene expression at the time of maturation in the two fixed size classes (large vs. small males), and (2) investigate whether social environment during ontogeny influences differential gene expression in intermediate males with more plastic mating strategies. Identification of the genes, pathways and environmental influences regulating the developmental age and size of males at sexual maturity and their size-associated mating behavior repertoire provides a key step in understanding the molecular genetic mechanisms leading to the maintenance of alternative mating strategies as evolutionary innovations.

STUDY SYSTEM

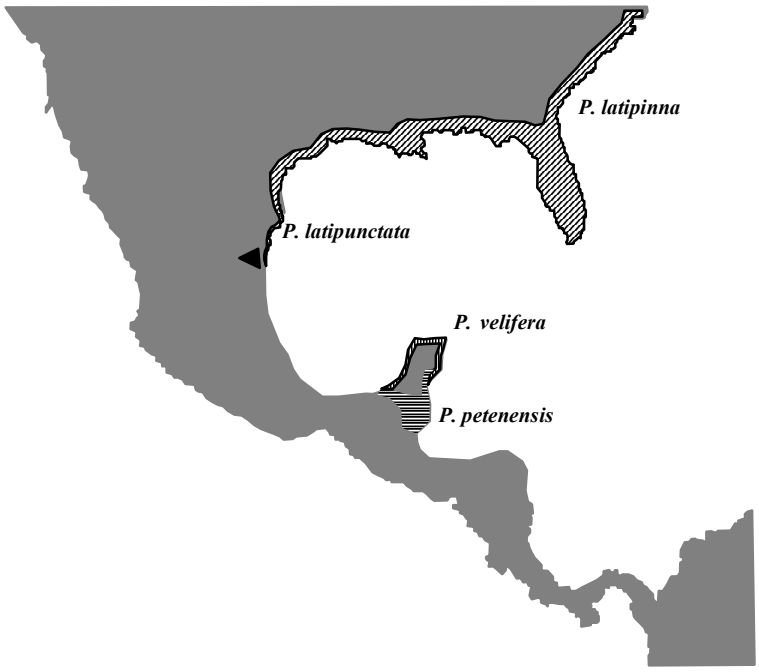

Figure 1.1. Current distribution of sailfin molly species

Sailfin mollies are in the livebearing fish family Poeciliidae and form a monophyletic lineage within the genus *Poecilia* (subgenus *Mollienesia*) that consists of

four species: *P. latipinna, P. latipunctata, P. velifera* and *P. petenensis* (Ptacek & Breden 1998). All species within the genus are found in saltwater marshes or freshwater ponds along the southern portions of the Atlantic and Gulf coasts of the United States and Mexico and into Belize and Guatemala (Figure 1.2; Ptacek & Breden 1998). In particular, the ability of *P. latipinna* to tolerate a wide range of salinities (0-35 ppt) and inhabit numerous habitats from inland freshwater ponds to saltwater estuaries allows them to have the largest geographic range of all the sailfin molly species (Rosen & Bailey 1963). *Poecilia latipinna* occurs from the Atlantic coast as far north as Georgetown, South Carolina and continues southward all along the Gulf coast into northern Mexico (Figure 1.1, Ptacek & Breden 1998).

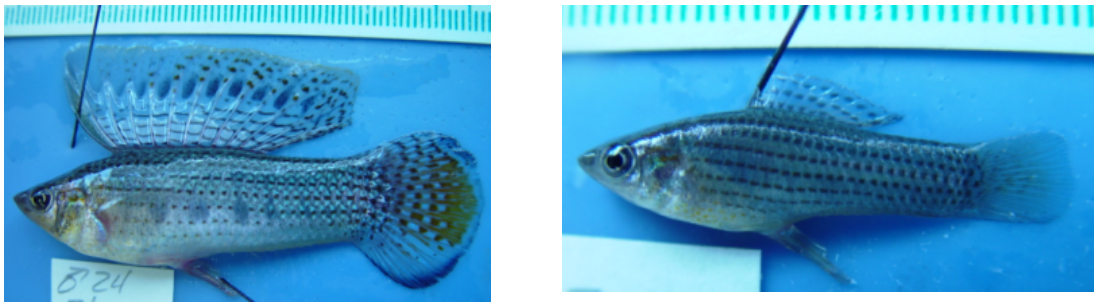

Figure 1.2. A large male (SL=50 mm; left) and a small male (SL=25 mm; right) *P. latipinna* from Steve's Ditch, Wakulla County, FL. Both males are sexually mature, as indicated by the fully fused gonopodium of each male.

Male sailfin mollies exhibit large variation in standard length (SL) both within and between populations (Figure 1.2; SL—measured from the tip of the mouth to the end of the caudal peduncle; Ptacek & Travis 1996; Seda *et al.* 2012). Male size at maturity is determinate and partially Y-linked (Kallman 1989; Travis 1994). The social environment

in which *P. latipinna* develop as juveniles is variable. Population size and large/small male ratios fluctuate seasonally over the course of the year (Figure 1.3). Females, however, have indeterminate growth and older females are often considerably larger than males (Ptacek & Travis 1996). Sailfin mollies are sexually dimorphic, with large and intermediate males having an enlarged dorsal fin (i.e., sailfin) used for courtship displays. Males of all sizes possess a modified anal fin (the gonopodium) that serves as a copulatory organ that transmits sperm to the female during internal fertilization (Farr 1989; Constanz 1989). Females have a brood of live fry approximately every 28 days (Trexler 1989). Shortly after birth, females are the most receptive to courtship and subsequent insemination, and advertise their receptivity by releasing pheromones in their urine (Travis & Woodward 1989). Females will cooperate in mating by remaining stationary after courtship displays, therefore facilitating successful sperm transfer by the male's gonopodium (Farr & Travis 1986; Farr 1989).

Female mating preferences place strong sexual selection pressures on male body size, dorsal fin size and courtship display rate in sailfin mollies. When given the choice, females will prefer the largest male (Ptacek & Travis 1997; Gabor & Page 2003; MacLaren *et al.* 2004). Female *P. latipinna* prefer males that appear larger, specifically those that have a larger total lateral projection area, the sum of the dorsal fin, caudal fin, and body areas (O'Brien et al 1976, 1985; Rowland 1989a,b; MacLaren *et al.* 2004). This preference for large males is particularly strong when the females are receptive either as a newly matured virgins or within 48 hours following parturition (Ptacek & Travis 1997; Gabor & Page 2003). Phylogenetic evidence suggests that female preference for large

sailfin size evolved before the evolution of the male sailfin trait itself (Ptacek 1998; Ptacek *et al.* 2011).

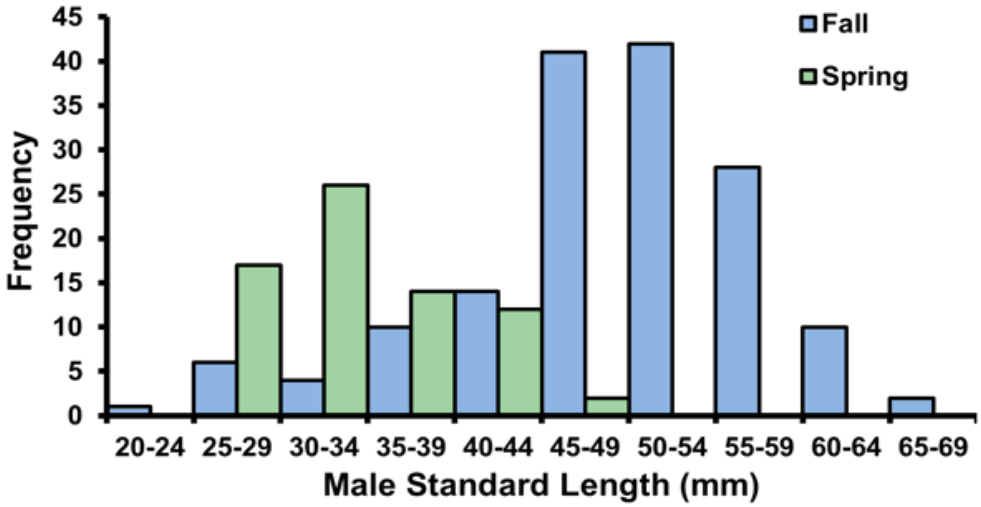

Figure 1.3: Number of small and large males found seasonally in Steve's Ditch population, Wakulla County, FL

Male body size not only affects female preference but also determines the alternative mating strategy used by the individual. Large males (larger than 44 mm) court females in a ritualized fashion by displaying their large colorful dorsal fin and swimming in a C-shape (Ptacek & Travis 1996; Seda *et al.* 2012). Smaller males (smaller than 30 mm),"sneakers", force copulation by forced insemination with gonopodial thrusts (Farr 1989). Intermediate-sized males (30 mm- 44 mm) are more plastic in their behavior and will switch from courting to sneaking behavior based on the social environment of competitor males (Travis & Woodward 1989). Although male mating behavior can be placed into discrete categories, the SL of male *P. latipinna* shows a continuous, normal

distribution indicative of a quantitative genetic trait under the influence of both genetics and the environment (Cummings 1943; Ptacek & Travis 1996).

Both sexual and natural selective pressures maintain the variation in size and mating behavior across populations of sailfin mollies (Ptacek 2005). Small males reach sexual maturity faster and therefore can spend most of their lifetime pursuing reproductive success (Travis 1989; Travis 1994). Large males mature much more slowly, nearly three times as long a juvenile period, but have greater mating success once mature since females have strong preferences for larger males (Ptacek & Travis 1997; MacLaren *et al.* 2004). Overall survival of mollies is size dependent, with larger individuals having lower mortalities (Trexler *et al.* 1992). However, small individuals are able to hide more easily from avian predators such as herons (Trexler *et al.* 1994). This balance between sexual and natural selection is thought to maintain the polymorphism in male size at maturity observed in natural populations (Travis 1994; Ptacek 2005).

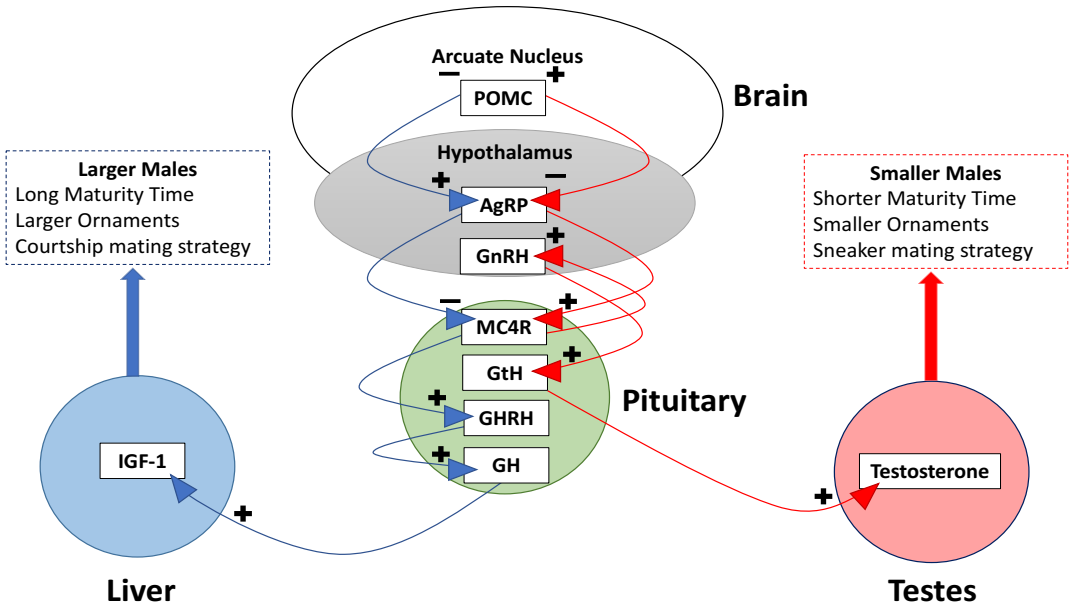

Figure 1.4. Model of HPG axis interactions and regulatory impact of size at maturity in male *P. latipinna* (modified after Zhang *et al.* 2012).

In males of *P. latipinna,* size at maturity, and therefore mating strategy is dependent on the length of juvenile development time before initiating sexual maturity. Males of *P. latipinna* show a wide range of time to maturation, from 25 to 200 days (Travis 1989). Small males mature more quickly (25-75 days) while large males can take up to a year to mature (Cummings 1943; Travis 1989). A small male will never attain the size of a large male, as further growth ceases once sexual maturity is reached (Figure 1.2). Maturity is complete when the gonopodium fully fuses (Cummings 1943; Constantz 1989), a process which is testosterone dependent. In male and even female poeciliids, a sustained increase of testosterone during development has been shown to be sufficient to initiate the molecular pathway toward forming a gonopodium (Angus *et. al* 2001). The hypothalamic-pituitary-gonadal (HPG) axis is the pathway that initiates this fusion and

subsequent sexual maturity (Kallman & Borkoski 1978; Zimmerer & Kallman 1989; Figure 1.4).

Sexual maturity is generally regulated by the integration of neuroendocrine and endocrine signals in the HPG axis along with environmental factors such as food availability, temperature, and the social environment during ontogeny (Schreibman and Kallman 1978; Duan 1997; Lampert *et al.* 2011). At the gonad level, spermatogenesis and gonopodium development is tightly regulated by stage and cell specific interactions of the expression of various hormones (Zimmerer & Kallman 1989). As mentioned above, the main endocrine regulators of gonadal maturation in males are steroid hormones such as testosterone and other androgens (Angus *et. al* 2001). However, sex steroids are not the only factors involved, because they interact with other upstream hormones in the brain and liver to influence sexual maturation (Figure 1.4). Within the arcuate nucleus of the brain, neurons secreting melanocortins (MC1-MC5), the products of the pro-opiomelanocortin (*pomc*) gene expressed in the hypothalamus, are activated by leptin (Schwartz *et al.*, 1997). These POMC neurons are stimulated during a positive energy balance (Schwartz *et al.*, 1997). AgRP, also released from the hypothalamus, is encoded by the *agrp* gene (Zhang *et al.* 2012). AgRP is an antagonistic protein to melanocortin 4 receptor (Mc4r), and participates in the regulation of energy homeostasis by blocking melanocortin signaling (Zhang *et al.* 2012). Transgenic zebrafish overexpressing *agrp* exhibited obesity and increased linear growth at the time of maturity (Zhang *et al.* 2012). Furthermore, Mc4r suppression by AgRP increased levels of growth hormone releasing hormone (GHRH) in the anterior pituitary. GHRH stimulates growth

hormone (GH) production and release, which is known to stimulate insulin-like growth factor-I (IGF-1; Reinecke 2010). Insulin and IGF-1 are mainly produced in the liver, which is the principal source of endocrine IGFs in fish (Reinecke 2010). Across taxa, animals with increased IGF-I levels show increased growth rates (Vasilatos-Younken & Scanes 1991). Serum IGF-I also provides negative feedback to the release of GH. Increased *mc4r* expression increases gonadotropin-releasing hormone (GnRH) secretion (Tao 2010). GnRH is released from the hypothalamus and promotes synthesis and release of the gonadotropin hormones (GtH) from the pituitary (Tao 2010). GtHs act on the gonads to promote the synthesis of steroid hormones (testosterone, 11-ketotestosterone and other androgens), which promote gonadal development, maturation and gametogenesis. In turn, these steroid hormones released from the gonads feedback to the brain to increase or decrease GtH release (Yaron *et al.* 2003; Zohar *et al.* 2010). Therefore, differences observed between large and small sailfin molly males are at least partially due to differential gene expression and possible regulatory interactions of the HPG axis (Figure 1.4; Table 1.1).

Table 1.1: Candidate Genes Associated with HPG Axis

	Expression levels		
Genes	Sneaker	Courter	Citation
pomc	high	low	Zhang *et al.* 2012
agrp	low	high	Zhang *et al.* 2012
mc4r	high	low	Lampert *et al.* 2010
ghrh	low	high	Reinecke 2010
gh	low	high	Reinecke *et al.* 2005
igf1	low	high	Reinecke 2010
gnrh	high	low	Smith *et al.* 2004
hsd17b3	high	low	Smith *et al.* 2004

Moreover, neural plasticity and neural signaling genes are also implicated in the developmental divergence and social status of males in multiple fish species with alternative mating tactics. In prior studies of Atlantic salmon, genes involved in synaptic function and plasticity like neuronal pentraxin, synaptotagmin, MHC class I proteins and ependymin (Table 1.2) were all highly expressed in sneaker males compared with immature males (Aubin-Horth 2005a). Interestingly, in a subsequent study examining the influence of social experience on male gene expression patterns, the authors found no difference in transcription of these genes between the brains of sneaker males and immature males sharing the same environment (Aubin-Horth 2005b). In several fish species, the neuropeptide arginine vasotocin (*avt*), is highly correlated with aggression and territoriality. Elevated expression of *avt* was detected in the territorial fish brain in the peacock blenny (*Salaria pavo*; Grober *et al.* 2002), African cichlid (*Astatotilapia*

burtoni; Greenwood *et al.* 2008) and three-spined stickleback (*Gasterosteus aculeatus*; Sanogo 2012). The neuropeptide somatostatin and somatostatin receptors have also been shown to play a role in social behavior of fishes (Trainor & Hofmann 2007) and somatostatin is a known inhibitor of growth hormone (Schunter *et al.* 2014), hence an important regulator of length of juvenile development time. In dominant cichlid males, the somatostatin prepropeptide and somatostatin receptor type 3 (*sstr3*) were elevated in comparison to the subordinate males. Brain aromatase is an important enzyme involved in the regulation of social status between many fish species. And finally, the *cyp19a1* gene encodes for both the brain and gonadal aromatase that breaks down androgens into estrogenic forms. Brain aromatase expression levels were found to be lower in castrated male Atlantic salmon (Mayer *et al.* 1991) and higher in individuals of Atlantic croaker (*Micropogonias undulates*) with fully developed gonads than in developing ones (Nunez *et al.* 2006).

In the case of my study species, *P. latipinna*, a prior study found transcripts upregulated in sneaker (non-dominant) males that were significantly enriched for GO terms associated with neurotransmission, learning and locomotor behavior (Fraser *et al.* 2014). Furthermore, in intermediate courting males, upregulated transcripts were significantly enriched for GO terms associated with mRNA processing and translation (Fraser *et al.* 2014). However, this gene expression analysis only examined the GO term enrichment patterns and did not identify candidate genes associated with alternative mating strategies and social environment differences. My thesis research focuses on measuring differential expression of genes at the time point of sexual maturity in males,

comparing (1) the two different fixed mating strategies (i.e. small sneaker vs. large courter males) and (2) the influence of social rearing environment on males of the plastic mating strategy (i.e. intermediate males) to determine possible candidate genes that explain variability in body size and size-associated alternative mating strategies in sailfin mollies.

Table 1.2: Candidate Genes Associated with Neural Plasticity and Neural Signaling

	Expression levels		
Genes	Sneaker	Courter	Citation
nptx	high	low	Aubin-Horth *et al.* 2005
syt	low	high	Aubin-Horth *et al.* 2005
mc4r	high	low	Lampert *et al.* 2010
ghrh	low	high	Reinecke 2010
avt	low	high	Reinecke *et al.* 2005
sst1, sst2	low	high	Reinecke 2010
cyp19a1	high	low	Smith *et al.* 2004

GOALS AND OBJECTIVES OF MY STUDY

My thesis research aimed to describe the male brain transcriptome of *P. latipinna* and to investigate the degree to which genes (G), social environment (E) during juvenile development, and developmental plasticity (GxE) influence alternative mating strategy expression and DE of genes at the time of sexual maturity.

To address these questions, I conducted a full sibling split brood design in which I reared full siblings split between three social conditions (3 females only, 2 females and 1 large male, 2 females and 1 small male) to investigate how (1) genes and their expression differences are associated with alternative fixed mating strategies, and (2) genes and their expression differences are associated with alternative plastic mating strategies as a result of different social environments experienced during juvenile development to sexual maturity.

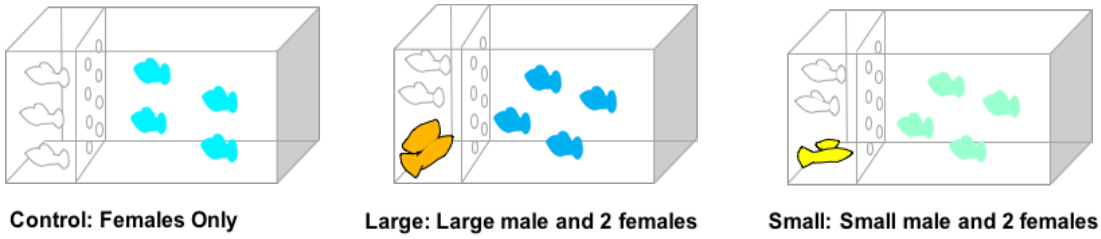

Figure 1.5. Rearing design of three experimental social ontogeny treatments.

Males used as sires in the breeding design were randomly chosen from a brackish water population (Steve's Ditch, Wakulla County, Florida) and mated with lab-reared virgin females. To examine the effect of sire size on male offspring size at maturity, wild caught males were divided into two major size classes: large (>48 mm) and small (<30

mm; Seda 2010; Seda *et al.* 2012). Five sires were used from the large size class and four from the small size class, resulting in nine families reared in the experimental breeding design. The five largest (range: 60 mm - 68 mm) and four smallest (range 27 mm - 29 mm) individuals were used as large and small sires, respectively. Sires from each size class were mated to virgin females. Males and females used as sires and dams were not known sibs. Females were housed in separate 18.9 L aquaria and since female mollies can store sperm for up to 6 months (Bisazza 1993), only virgins were used as dams to ensure sire paternity of all experimental offspring. Once the inseminated females from each family gave birth, the offspring were divided between the three social ontogeny treatments with six siblings in each treatment. The three social treatments (Figure 1.5) during ontogeny were large (one large male and two females), small (one small male and two females) and females (three females). Adults and offspring were separated in the tanks by a plastic barrier with perforations to allow visual, chemical, and hormonal signals to be transmitted from one side of the tank to another, but to exclude adults from food provided for the juveniles. At maturity—determined by the fusion of the anal fin to form the gonopodium (Cummings 1943)—male offspring were photographed (Canon Rebel XSi), weighed to the nearest 0.001 g, and measured to the nearest mm for SL. Males were euthanized (ice bath) and preserved for genetics (brain dissected but left within brain case and perfused in RNAlater for RNA extraction; samples stored at -80C). Behavioral profiles were not measured since previous research has shown that there is a strong correlation between SL and the rates of certain mating behaviors in the Steve's

Ditch population and we wanted to control the time point of sexual maturity for all males used in the gene expression profiles (Ptacek & Travis 1996, Seda et. al 2012).

For RNA extraction samples were removed from the -80C freezer and homogenized to a fine solution using Omni TH (Omni International, GA, USA) tissue homogenizer. Total RNAs were extracted from each brain sample using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was assessed by Qubit (Thermo Scientific, Wilmington, Germany) and RNA integrity (RIN) was assessed using a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only brain samples with RNA extraction RIN scores of > 8 were used in RNA-Seq libraries.

RNA-Seq library preparation and Illumina sequencing were done at Florida State University (Biological Sciences and College of Medicine molecular analytical facilities). To obtain high-throughput brain transcriptome data of sailfin mollies, complementary DNA (cDNA) libraries were prepared using a NEBNext Multiplex Small RNA library prep kit (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. We conducted 150 bp paired-end sequencing on nine samples and 100 bp single-end sequencing on 36 samples using an Illumina HiSeq2500 (Illumina, San Diego, CA, USA). The nine paired-end samples were used only in the construction of the transcriptome. After sequencing, overall sequence quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were trimmed using ConDeTriV2.2 (Smeds & Kunstner, 2011). RNA-Seq data from 36 individuals was pooled and then de novo assembled using Trinity (version 2.4.0; Grabherr *et al.*, 2011)

with default values. Blast2GO Pro (version 4.1; Conesa *et al.* 2005) was applied for the homology search to predict the function of unigenes. The function of unigenes was predicted using Blastx to search all possible proteins against the NCBI Non-redundant (NR) database (accessed on 11 November 2017). The criterion regarding significance of the similarity was set at Expect-value less than 1×10^{-5}. Functional signatures were searched using InterProScan (Zdobnov & Apweiler 2001), the KEGG database (Kanehisa 2002) and the Gene Ontology database (Harris *et al.* 2004).

Bowtie2 (version 2.1.0; Langmead and Salzberg 2012) was used to multi-map reads to the *de novo* transcriptome assembly before clustering the transcripts with Corset v.1.03 (Davidson and Oshlack 2014). Differences in gene expression for all transcripts were analyzed from raw read counts using the R (R Development Core Team 2015) package DESeq2 (version 3.6; Love *et al.* 2014). Analyses calculated log2FoldChanges (LFC) per gene as a function of sire size, social environment, and their interaction. Statistical significance was determined based on the false discovery rate (FDR) adjusted p-values <0.05 (Benjamini and Hochberg 1995). Gene set enrichment analysis (GSEA v.2.1.0) was also performed to identify pre-defined gene sets that showed significant, concordant differences in expression between treatment groups and alternative mating strategies (Subramanian *et al.* 2005).

The results of my thesis research (found in Chapter 2) will contribute to our understanding of *P. latipinna* gene expression patterns and alternative male mating strategies. Understanding how alternative mating strategies arise and are subsequently maintained in the genome is crucial to understanding evolutionary phases leading to

speciation. In addition, I have assembled the first described brain transcriptome for males of *P. latipinna*. This transcriptome will be a valuable resource for poeciliid research and adds to a growing body of study on organismal genetic diversity.

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CHAPTER TWO

THE ROLE OF SOCIAL ENVIRONMENT AND GENOMIC PLASTICITY IN THE MAINTENANCE OF ALTERNATIVE MATING STRATEGIES IN SAILFIN MOLLIES

BACKGROUND

How phenotypic variation is maintained in the face of strong natural and sexual selection is a key paradox in evolutionary biology, as genetic variability should be lost in response to selection favoring locally adaptive phenotypes (Lewontin 1974; Charlesworth & Hughes 2000; Mitchell-Olds *et al.* 2007). One solution to this paradox that is particularly important for traits associated with reproductive fitness, is phenotypic plasticity, the ability of a single genotype to express different phenotypes in response to varying environmental cues (West-Eberhard 2003; Auld, Agrawal & Relyea 2009; Scheiner & Holt 2012). Such plasticity in condition-dependent reproductive traits has led to the expression of alternative reproductive strategies or tactics in a number of animal species (Gross 1996; Brockmann 2001; Neff & Svensson 2013). While numerous studies have examined how alternative mating strategies are maintained through fitness differences in morphological, behavioral and physiological traits as a result of some form of balancing selection (e.g., negative frequency dependent natural or sexual selection), we still know little about the underlying genetic basis of these plastic responses or how changes in genomic architecture can favor the evolution of adaptive plasticity in life history and reproductive traits (Wimberger 1992, Rousseau & Dufour 2007).

Understanding how reproductive plasticity arises and is subsequently maintained in the genome is crucial to understanding evolutionary phases leading to speciation, as the maintenance of polymorphism in mating strategies can influence how quickly populations can diverge (Coyne and Orr 1998). When novel alternative phenotypes arise within a population they can go to fixation leading to rapid population divergence or be maintained as polymorphisms, slowing the rate of divergence. Variable environmental conditions and underlying plasticity genotypes can allow for evolution of new specializations within the novel trait without elimination of the established trait and lead to the maintenance of variability in mating strategies within species (West-Eberhard 2003). Understanding how changes in the genome lead to developmental plasticity of life history and reproductive traits provides the basis for understanding the evolution of alternative mating strategies.

Teleost fishes provide some of the clearest examples of alternative mating strategies and phenotypic variation in reproductive traits (Taborsky 2001; Taborsky, Oliveira & Brockmann 2008Engagement in several alternative strategies may be fixed or variable within species across their reproductive lifespan (Taborsky 1994, 2001; Aubin-Horth *et al.* 2005, 2009). In some species, individual tactics are fixed and can occur either through inherited genetic polymorphisms (Lank *et al.* 1995; Schuster & Sassaman 1997; Kupper *et al.* 2016; Lamichhaney *et al.* 2016), conditional-dependent switches triggered before sexual maturation (Gross 1996; Taborsky 1998; Gross & Repka 1998) or a combination of genetic and environmental factors (Piche, Hutchings & Blanchard 2008; Neff & Svensson 2013). In other species, individual tactics are plastic throughout

reproductive life, in response to social or environmental cues (Gross 1996; Moore 1991; Taborsky, Oliveira & Brockmann 2008).

One common polymorphism seen among males in many fishes is the alternative mating strategy of 'courting' vs. 'sneaking' behaviors associated with male size at maturity (Gross 1996; Fukaya *et al*. 2009; Rios-Cardenas and Morris 2011). The variation observed in male size at maturity is usually a result of life history differences in the duration of the juvenile growth period and age at time of maturation (Gross 1996; Rios-Cardenas and Morris 2011). Early-maturing males are smaller and adopt a sneaker strategy, while older, later-maturing males are larger and perform courtship displays to attract females and/or maintain breeding territories (Constantz 1989; Farr 1989; Gross 1996). Numerous phylogenetic and behavioral studies have been conducted in fishes to study the evolutionary history and fitness consequences of alternative mating strategies (Hutchings & Myers 1994; Gross 1996; Taborsky, Oliveira & Brockmann 2008). However, far less is known about the genetic basis of these developmentally plastic traits (Fraser *et al*. 2014).

Social interactions can play critical roles in maintaining plasticity in life history and mating strategies (Kasumovic & Brooks 2011) and the fitness consequences of alternative mating strategies are often mediated by social environment (Rodd & Sokolowski 1995; Tudor & Morris 2009). Alternative mating strategies often lead to phenotypic dimorphism where males adopting different strategies differ in their degree of morphological ornamentation as well as behavioral tactic. Social interactions have been shown to trigger this behavioral and phenotypic difference (e.g. African cichlids,

Maruska & Fernald 2010) and appear to be particularly important in poeciliid fishes (Rodd, Reznick & Sokolowski 1997; Lutnesky & Adkins 2003; Walling *et al*. 2007; Magellan & Magurran 2009; Kasumovic & Brooks 2011; Rios-Cardenas & Morris 2011). For example, in green swordtails (*Xiphophorus helleri*) males matured later, with longer swords (sexually selected ornament), in response to visual cues of large males with greater sexual ornamentation in their environment during juvenile development (Walling *et al.* 2007). In guppies (*Poecilia reticulata*), juvenile males delayed maturation and matured at larger body size when exposed to social environments that had both female and male adults (Rodd, Reznick & Sokolowski 1997; Magellan & Magurran 2009). While these studies suggest that social interactions during ontogeny may be an important influence underlying developmental plasticity of life history and mating traits in male poeciliids, the genetic underpinnings of the development of alternative mating strategies in these fishes are unknown.

Advances in high throughput genomics have made it possible to begin to examine the molecular basis of behavioral variation among alternative phenotypes in fishes. Studies in fishes with alternative mating strategies have documented large expression differences in genes associated with feeding, reproduction and neural signaling (Aubin-Horth *et al.* 2005; Fraser *et al.* 2014; Schunter *et al*. 2014). In Atlantic salmon (*Salmo salar*), large males are territorial and anadromous. In the same population, sneaker males live out their lives in freshwater and sneak into the nest of a migrating female during egg laying (Rowe & Thorpe 1990). As in many fishes, the mating strategy is developmentally plastic; the type of behavior a male exhibits is determined by the size and resources

attained during the time period before reaching sexual maturity (Rowe & Thorpe 1990; Hutchings & Myers 1994; Thorpe 1994; Thorpe *et al.* 1998; Aubin-Horth & Dodson 2004). When gene expression levels were quantified between sneaker males and age-matched immature males (future large anadromous males), 15% of genes were differentially expressed (Aubin-Horth *et al*. 2005). These included genes involved in the endocrine reproductive pathway associated with maturation, and also genes related to learning and memory processes (Aubin-Horth *et al.* 2005). The genes related to cognition were upregulated in the sneaker males, however, it is unknown whether this change in learning and memory is related to the sneaking strategy or to maturation in general, since large mature males were not used in the study (Aubin-Horth *et al.* 2005). Interestingly, a similar pattern of large expression differences between alternative male strategies was also found in black-faced blennies (*Tripterygion delaisi*). More genes were differentially expressed between the territorial and sneaker male phenotypes than between males and females (Schunter *et al.* 2014), suggesting that reproductive phenotype is a more important factor in differential gene expression profiles than sexual dimorphism. However, unlike the findings in Atlantic salmon, territorial blenny males overexpressed genes related to synaptic plasticity while sneaker males overexpressed genes involved in differentiation and development (Schunter *et al.* 2014), suggesting that certain candidate genes in the context of alternative mating strategies may be species specific.

The live-bearing fish family Poeciliidae offers a number of examples of species with alternative mating strategies associated with male age and size at maturity (Rios-Cardenas & Morris 2011). Alternative mating strategies have arisen multiple times

independently in Poeciliidae, potentially promoting speciation in these fishes (Ptacek *et al*. 2011). Large male size, ornamentation and courtship display behavior are all targets of female mating preferences in poeciliids (Rios-Cardenas & Morris 2011) thus, the development of 'courter' versus 'sneaker' mating strategies is a likely target for modification as a result of social experience. Behavioral phenotypes may be genetically based, but developmental plasticity in the use of alternative mating behaviors could be mediated by interactions of those genes with social experience (visual, hormonal, or chemical) during juvenile development (Kasumovic & Brooks 2011).

The sailfin molly (*P. latipinna*), a common poeciliid fish of coastal marshes in the southeastern U.S., offers an exemplary model species for testing hypotheses regarding the role of genes and environment in the development and maintenance of alternative mating strategies. Male sailfin mollies exhibit the typical size-associated mating strategy polymorphism found in many poeciliids. Large males have enlarged dorsal fins (sailfin ornament) used in courtship displays while small males lack the morphological ornamentation and adopt a sneaker male strategy where males attempt to insert the modified anal fin, the gonopodium, into the female's gonopore without female cooperation in the mating attempt (internal fertilization is characteristic of most poeciliids; Farr 1989). Interestingly, male mollies, like most poeciliid species, have determinate growth (male size is fixed at sexual maturity) and size at maturity (and size-associated dorsal fin size) in natural populations shows a normal, continuous distribution, characteristic of the contribution of additive autosomal genes (Loveless *et al.* 2010; Seda *et al.* 2012). While evidence of Y-linked contributions to male size at maturity have been

shown (Travis 1994), environmental influences such as salinity, temperature, nutrient availability and social experience are also known to influence age and size at maturation as well (Trexler & Travis 1990; Trexler, Travis & Trexler 1990; Lange 2013). The continuous distribution of male sizes at sexual maturity results in small, large and intermediate-sized males. While small and large males are 'fixed' in their mating behavioral strategy (sneakers vs. courters), intermediate-sized males show much more behavioral flexibility (Farr, Travis & Trexler 1986; Ptacek & Travis 1996; Seda *et al.* 2012). Behavioral plasticity of intermediate males appears to be strongly influenced by the social environment of competitor males, whereby these males adopt a courting strategy in the absence of other males and a sneaking strategy in the presence of other males, particularly larger males (Travis & Woodward 1989; Fraser *et al.* 2014). Indeed, in a prior study of genomic plasticity between small and intermediate males, Fraser *et al.* (2014) found that behavioral plasticity between intermediate males in different social settings (alone vs. with competitor males) was accompanied by broader and more robust changes in gene expression than those found in small males exposed to these same social conditions. Results from both behavioral and genomic studies suggest, in sailfin mollies, the courtship/sneaking dichotomy is more strongly genetically determined between males at the extremes of the size distribution but more environmentally regulated in the intermediate size class (Fraser *et al.* 2014).

In non-model species like *P. latipinna*, it is difficult to study differential expression at the gene level in the absence of a quality reference genome (Scoville & Pfender 2010). Here we used RNAseq to first characterize the brain transcriptome of the

male sailfin molly and then to detect differentially expressed (DE) genes in the brain between males of different sizes at maturity, and hence, alternative mating strategies. We reared juvenile male mollies under three different social ontogeny treatments (females only, females and large male, females and small male) in order to test two hypotheses. First, small and large males with fixed alternative mating strategies show DE of genes known to influence sneaker vs. courtship strategies, respectively, regardless of social experience. Second, for intermediate-sized males, social experience during ontogeny influences plasticity in mating strategy as a result of differential gene expression in response to social experience during juvenile development. Here, we describe the *de novo* assembly of the male *P. latipinna* brain transcriptome and identify individual genes and gene sets whose expression changes in 1) small and large males with fixed alternative mating strategies regardless of social context and 2) intermediate males with plastic strategies in response to three different social ontogeny treatments experienced during juvenile development to sexual maturity.

METHODS

Experimental animals

We used wild-caught males and females of *P. latipinna* from a salt marsh population (Steve's Ditch) near Panacea, Florida, USA (N29°58.379', W084°23.357') to establish a breeding stock housed in 600 liter stock tanks in the Clemson University research greenhouse (Biosystems Research Center, Animal Use Protocol No. 2014-053).

Fish were kept year round at 12 ppt salinity, 25°C and ambient light conditions for Clemson, SC, USA and fed a commercial mix of shrimp and algae flaked food.

Social ontogeny rearing experiment design

The Steve's Ditch population was chosen as the source population for our social ontogeny experiment for three reasons. First, sampling and prior behavioral studies of this population for over 20 years show that males mature across a wide range of sizes at maturity (~20-65 mm standard length (SL); Ptacek & Travis 1996; Ptacek 2005; Seda *et al.* 2012). Second, repeated measures of mating behavioral profiles of males from this population show that courtship display rate is strongly, positively allometrically associated with male SL (e.g., slope = 4.44, $P < 0.05$; Seda *et al.* 2012) and negatively allometrically associated with male SL for gonopodial thrust rate (forced copulation attempts of sneaker males; e.g., slope = -7.03, $P < 0.05$; Seda *et al.* 2012). Finally, a repeated pattern of seasonal variation in male size at maturity exists in this population, with small males occurring at high frequency in early spring (March – June), being replaced by predominantly intermediate and large males by late summer and early fall (July – November); few adult males survive winter conditions in north Florida (Trexler & Travis 1990; Travis, Ptacek & Lange, pers. obs.). Thus, social experience with adult males of different sizes changes across the breeding season for maturing males in this wild population.

Wild caught large males and laboratory-reared small males and virgin females were used as sires and dams in our social ontogeny experiment. While male size at maturity in this population approximates a normal distribution, we categorize males used

as sires and male offspring reared to maturity in our study into three size classes based upon the total variance in male size in Steve's Ditch: small males, 21 – 29 mm SL; intermediate males, 30 – 44 mm SL; large males, > 44 mm SL. Similar size ranges for the three male size categories have been used by previous behavioral and genomic studies of north Florida populations of sailfin mollies (Travis & Woodward 1989; Seda *et al*. 2012; Fraser *et al.* 2014).

Six large males and four small males were mated to virgin females and at parturition, broods were divided into three social ontogeny treatment rearing aquaria (18.59 liter): females: social group of three adult females; large male: social group of one adult large male and two adult females; small male: social group of one adult small male and two adult females. Adults and offspring were separated in the tanks by a clear, plastic barrier with perforations to allow visual signals including mating behaviors in adults, and chemical, particularly hormonal, signals to be transmitted from one side of the tank to the other, but to exclude adults from food provided for the juveniles. The social ontogeny experiment was carried out in the Aquatic Animal Research laboratory at Clemson University (Animal Use Protocol # 2014-029) where fish were reared at 12 ppt salinity, under controlled conditions (25°C, 14:10 h light/dark cycle) and fed commercial flaked food ad libitum twice daily.

Juveniles were reared in one of the three social ontogeny treatments until they reached sexual maturity. Maturity in males is reached when the anal fin rays fuse to form the gonopodium, the fin used in sperm transfer to the female's gonopore during internal fertilization characteristic of poeciliid fishes; male offspring were sacrificed on the day

when development of the sheath at the tip of the gonopodium was visually determined to be complete (Constanz 1989), always at the same time of day (between 1300 and 1500 hrs) to control for circadian rhythm and other diel differences among individuals. Length of juvenile development period (age in days at maturity) and male SL (mm) were recorded for each experimental male upon maturity. The effects of sire size and social ontogeny treatment on the age and size at maturity of experimental males were evaluated through two-way factorial ANOVA.

Whole brain tissue extracted at the time of maturity was used for transcriptome development. Following euthanasia in cold water of each mature male offspring reared in the social ontogeny experiment, a coarse dissection removed the braincase, which was placed in RNAlater, stored in 4°C for 24 h, followed by storage at -80°C. All animal handling and euthanasia procedures were carried out in accordance with the Clemson University Institutional Animal Care and Use Committee.

RNA extraction and sequencing

Whole brains were dissected from course dissections of experimental male fish stored in RNAlater at -80°C. Total RNA was extracted from homogenized brains using RNeasy Lipid Tissue Mini Kit (Qiagen) according to manufacturer's recommendations. RNA quality was determined using an Agilent Bioanalyzer system (Agilent Technologies) and only samples with an RNA Integrity Number (RIN) greater than 8.0 were used for RNA library construction. RNA-seq libraries were constructed from a total of 36 brains of mature males reared in the social ontogeny experiment using NEBNext® Ultra™ RNA library prep kit for Illumina (New England Biolabs) following

manufacturer's specifications, in the Bioanalytical and Molecular Core Facility, Department of Biological Sciences, Florida State University, Tallahassee, FL. Libraries were then sequenced using the Illumina HiSeq 2500 platform (100 bp, paired end reads) in the Translational Science Laboratory in the College of Medicine, Florida State University, Tallahassee, FL. Sequencing generated over 956 million raw 100-bp reads, with an average of 28.2 million reads per sample. Resulting read quality was assessed with FastQC v.0.11.3, followed by adaptor trimming with Trimmomatic (Bolger *et al.* 2014) and content-dependent quality trimming with default settings of ConDeTri (Smeds and Kunster 2011). The average per-read PHRED quality score after trimming and filtering was 37.

Transcriptome Assembly and Annotation

Trimmed and filtered reads from all samples were combined for *de novo* transcriptome assembly on the Palmetto high performance computing cluster at Clemson University using the Trinity v.2.4.0 pipeline with default parameters (Grabherr *et al.*, 2011). Assembled transcripts were annotated with Blast2GO (version 4.1; Conesa *et al.* 2005) which executes a BLASTx search against the NCBI nonredundant (nr) database and assigns gene ontology (GO) terms, Interpro IDs, enzyme codes and KEGG pathways to sequences with at least one significant BLASTx hit ($E < 1.0$ e-6).

Differential Gene Expression Analysis

Transcriptome data were used to identify DE genes between (1) large and small males pooled across all social ontogeny treatments, and (2) intermediate males raised in the three different social ontogeny treatments in pairwise comparisons. In both cases,

gene expression was quantified by mapping reads from the relevant samples back to the newly-assembled sailfin molly transcriptome using RSEM (Li and Dewey 2011). Reads from all splice forms of a given gene were pooled for downstream analysis. Count data were imported to DESeq2 for DE analysis using default parameters (version 3.6; Love *et al.* 2014). Initial exploratory analysis showed clear clustering by sire identity so every comparison contained sire identity as part of the DESeq2 design model. For comparison (1) between small and large males, size class and sire identity were included in the model. For comparison (2) among intermediate males, both social ontogeny treatment and sire identity were included in the model.

Gene set enrichment analysis (GSEA v.2.1.0) was also performed to identify pre-defined gene sets that showed significant, concordant differences in expression between size and social ontogeny treatment groups (Subramanian *et al.* 2005). A custom GSEA database of 940 gene sets, each containing at least 15 genes, was created from GO terms and enzyme code annotations of the sailfin molly transcriptome. Gene sets whose expression was enriched or depleted were identified using FDR=0.05.

RESULTS

Sire size and social ontogeny treatment influences on size or age at maturity

A total of 41 juveniles matured as males in our social ontogeny breeding design (N = 7 small males, N = 25 intermediate males, N = 9 large males). A two-way ANOVA was conducted to test for the effects of sire size, social ontogeny treatment and their interaction on the life history traits of male size (SL in mm) and age (days of juvenile

development) at maturity. Sire size included two levels (small and large), based on the three established categories of male body size. Social ontogeny treatment consisted of three levels (females, large male, small male: females, N = 12 males; large, N = 13 males; small, N = 16 males). Results of the ANOVA for male SL at maturity yielded no significant main effects of sire size ($F=0.395$ $p=0.534$) or social ontogeny treatment ($F=0.470$, $p=0.629$). The interaction term between sire size and social ontogeny treatment for SL at maturity was also not significant ($F=0.377$, $p=0.861$). The 41 male offspring reared in the social ontogeny experiment ranged in SL at maturity from 22 – 58 mm. The greatest variance in male size at maturity (females=105 mm; large=66.5 mm; small=30.8 mm) was found for males reared in the females social ontogeny treatment, while the least variance and largest median SL (females=36.5 mm; large=36.7 mm; small=42.0 mm) at maturity was found for males maturing in the small male social ontogeny treatment (Figure 2.1). In addition, results of the ANOVA for age at maturity also yielded no main effects of sire size ($F=1.14$, $p=0.293$) or social ontogeny treatment ($F=0.698$, $p=0.505$) nor of their interaction ($F=0.637$, $p=0.673$).

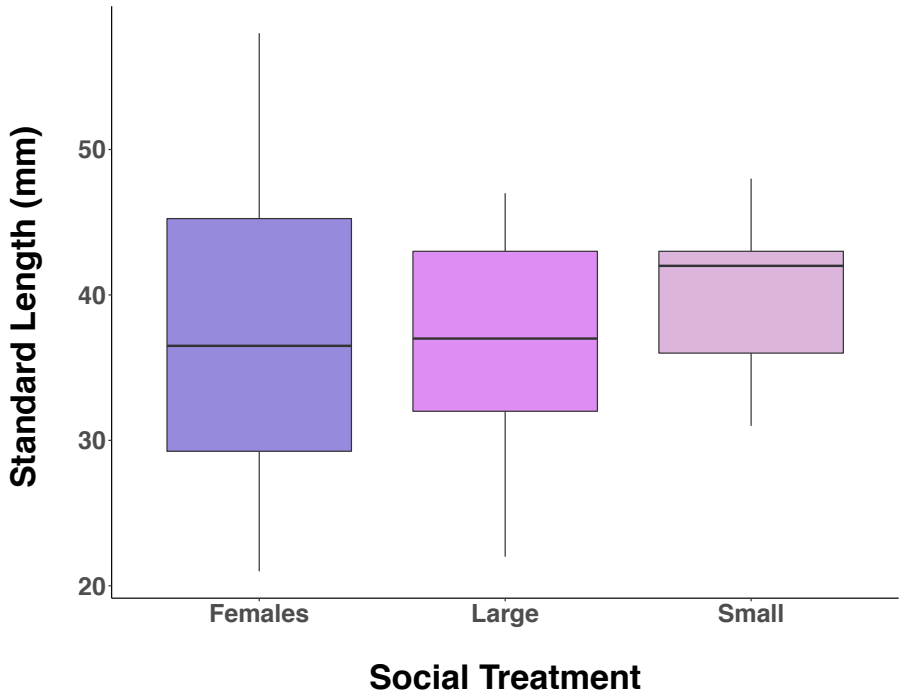

Figure 2.1. Standard length of males at maturity categorized by social ontogeny treatment during juvenile development to maturity. Boxes represent 75th and 25th percentiles (interquartile range) and whiskers represent 90th and 10th percentiles. The solid line is the median. Social ontogeny treatment: females, N = 12 males; large male, N = 13 males; small male, N = 16 males.

Transcriptome Assembly

Transcriptome sequencing and *de novo* assembly Illumina sequencing of 36 RNA samples yielding RIN scores > 8.0 from the 41 mature males reared in the social ontogeny experiment (N = 12 males from each social rearing condition treatment) generated over 956 million raw 100-bp reads, with an average of 28.2 million reads per sample. After filtering and trimming, clean reads from all samples were used for *de novo* transcriptome assembly with the Trinity pipeline. The male sailfin molly brain

transcriptome consisted of 277,718 high quality contigs, an overall N50 of 1249 bp, and a mean length of 729 bp.

Of the 36 mature male RNA samples used to construct the whole brain transcriptome, we excluded nine samples (8 intermediate and 1 small male) from subsequent DE gene analyses. Total RNA of these samples was extracted by one of us (MBP) nearly two years (Oct. 2015) earlier than the remaining 27 brain samples (extracted Feb. 2017) and used a different RNeasy Lipid Tissue Minikit that was nearing its expiration date. These nine brain samples showed widely different expression patterns from the other 27 male brains in our data set, causing up to 91% variance across PC1 in diagnostic PCA analysis of rlog data and disrupting the Cooks distance cutoff parameters within DESeq2. Due to their difference in RNA extraction procedures and storage time of their RNA we felt justified in excluding these 9 brain samples from DE gene analysis.

Overall differential gene expression and gene set enrichment results between males of large and small fixed mating strategies

To examine DE of genes between small and large males with fixed alternative mating strategies, we examined transcriptional differences between large (>44 mm, N = 9) and small males (<30 mm, N = 3) pooled among social ontogeny treatments. Recall that behavioral differences between small and large males persist in a common laboratory environment (Farr, Travis & Trexler 1986; Ptacek & Travis 1996; Ptacek 2005; Seda *et al.* 2012) and are therefore most likely regulated by divergent genotypes (Fraser *et al.* 2014).

We found a total of 1778 protein coding genes that were differentially expressed (DE) between small males and large males (FDR=0.05). The majority of these genes (1212) were up-regulated in small males with moderate fold changes. The remaining genes (566) were up-regulated in large males, generally with higher fold changes. As expected, both significant expression patterns and hierarchical clustering (seen above the heatmap in Figure 2.2) showed differentiation between small and large males, but not among social ontogeny treatments. This suggests that social ontogeny has a negligible effect on gene expression patterns in the fixed mating strategies. The top 20 annotated up-regulated genes sorted by p-value in small and large males are provided in Table 2.1 and 2.2.

In parallel with the identification of individual DE genes, GSEA was used to identify predefined gene sets whose expression differed between small and large males (FDR = 0.05). While DESeq2 identifies individual genes with large significant fold changes, GSEA identifies gene *sets* whose individual members show concordant, but potentially smaller, differences in expression (Subramanian *et al.* 2005). We found 365 gene sets that were significantly enriched in the small males (FDR=0.05). Large males had only 11 gene sets that were significantly enriched. The top 20 DE gene sets enriched in small males and the 11 gene sets enriched in large males are shown in Tables 2.3 and 2.4, respectively.

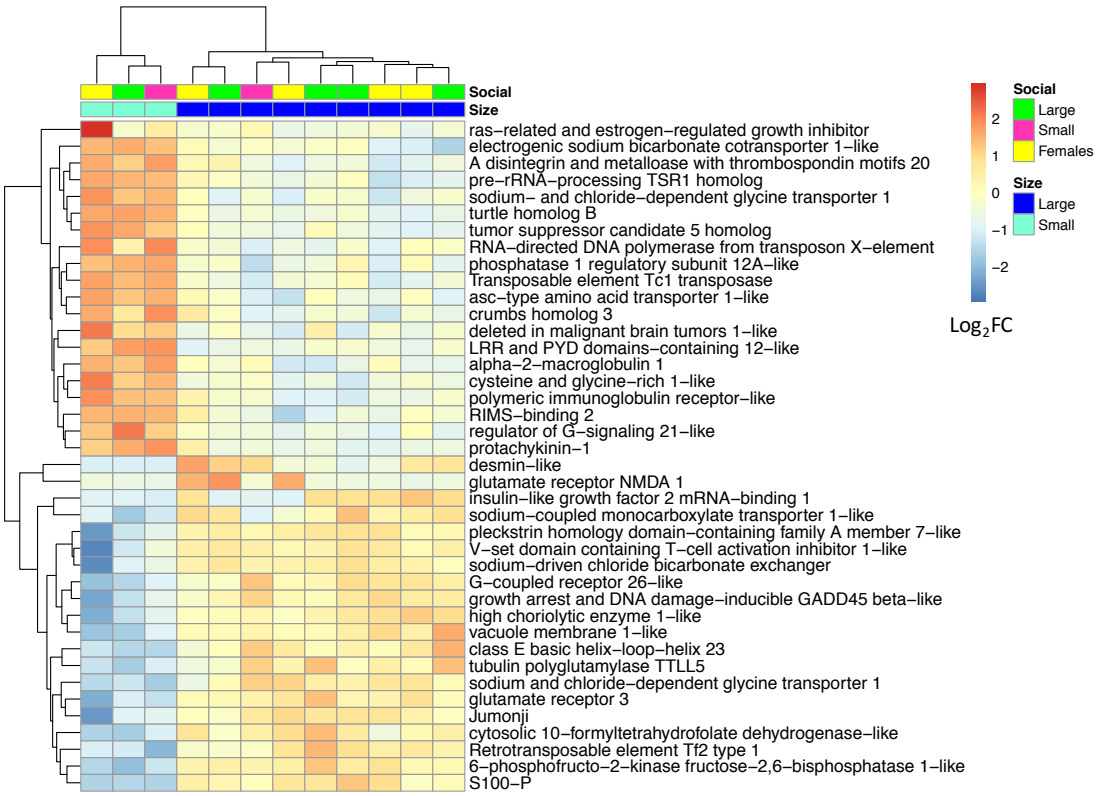

Fig. 2.2 Heatmap of Log2FC estimates and sample clustering for the top 20 differentially-expressed genes sorted by p-value in small and large males. Similarity between individuals with hierarchical clustering can be seen above the heatmap. Orange and blue indicate up-and down-regulation, respectively. Log2FC: Log2FoldChange.

Table 2.1. Mean normalized read counts, fold changes and FDR-adjusted P-values associated with 20 genes upregulated in small males whose expression differed significantly between small and large males.

Sequence ID	Gene	Small Male	Large Male	Fold Change	P-*adj*
TRINITY_DN31827_c4_g3	*mGlu3*	868	414	2.10	0.000
TRINITY_DN32016_c4_g4	*igf2bp2*	146	10	14.60	0.000
TRINITY_DN30765_c4_g2	*ppp1R12a*	121	33	3.67	0.000
TRINITY_DN32793_c2_g4	*aldh1l1*	1089	472	2.31	0.000
TRINITY_DN29813_c3_g4	*slc4a4*	61	9	6.78	0.000
TRINITY_DN33092_c1_g5	*plekha7*	156	52	3.00	0.000
TRINITY_DN30296_c3_g4	*pfkfb1*	122	13	9.38	0.000
TRINITY_DN32597_c2_g1	*slc6a9*	297	81	3.67	0.000
TRINITY_DN35971_c1_g2	*rimbp2*	791	400	1.98	0.000
TRINITY_DN27860_c5_g4	*slc7a10*	125	37	3.38	0.000
TRINITY_DN29683_c5_g2	*adamts20*	350	115	3.04	0.000
TRINITY_DN29236_c5_g5	*slc6a9*	152	29	5.24	0.000
TRINITY_DN31045_c0_g6	*hcea*	102	12	8.50	0.000
TRINITY_DN29767_c2_g2	*pol*	164	36	4.56	0.000
TRINITY_DN31565_c1_g1	*tc1a*	39	5	7.80	0.000
TRINITY_DN32264_c3_g3	*dmbt1*	13	1	13.00	0.000
TRINITY_DN36593_c2_g2	*tf2-4*	764	63	12.13	0.000
TRINITY_DN34106_c3_g3	*jardi2*	6997	804	8.70	0.000
TRINITY_DN29631_c6_g1	*a2m*	616	156	3.95	0.000
TRINITY_DN36227_c7_g2	*tutlB*	923	484	1.91	0.000

Table 2.2. Mean normalized read counts, fold changes and FDR- adjusted *P*-values associated with 20 genes upregulated in large males whose expression differed significantly between small and large males.

Sequence ID	Gene	Small Male	Large Male	Fold Change	P-*adj*
TRINITY_DN29279_c2_g1	*tusc5*	31	103	3.32	0.000
TRINITY_DN25223_c0_g1	*gpr26*	140	462	3.30	0.000
TRINITY_DN29077_c5_g3	*nlrp12*	117	475	4.06	0.000
TRINITY_DN32066_c8_g1	*s100p*	218	726	3.33	0.000
TRINITY_DN33192_c5_g1	*tsr1*	58	243	4.19	0.000
TRINITY_DN35288_c3_g1	*vcn1*	0	41	41.0	0.000
TRINITY_DN30175_c1_g1	*csrp1*	105	419	3.99	0.000
TRINITY_DN29396_c2_g5	*rgs21*	31	75	2.42	0.000
TRINITY_DN29582_c5_g2	*vmp1*	629	1276	2.03	0.000
TRINITY_DN35593_c5_g4	*bhlhe23*	112	297	2.65	0.001
TRINITY_DN32507_c1_g3	*crb3*	13	40	3.08	0.001
TRINITY_DN34467_c4_g1	*des*	33	160	4.85	0.001
TRINITY_DN33345_c0_g11	*ttll5*	12	47	3.92	0.001
TRINITY_DN36131_c17_g1	*grin1*	3213	5676	1.77	0.001
TRINITY_DN32670_c6_g1	*slc4a10*	235	743	3.16	0.001
TRINITY_DN31155_c4_g3	*tac1*	534	1220	2.28	0.001
TRINITY_DN31072_c6_g7	*gadd4b*	12	45	3.75	0.002
TRINITY_DN34337_c4_g2	*pigr*	1	46	46.0	0.002
TRINITY_DN7407_c0_g1	*rerg*	12	55	4.58	0.002
TRINITY_DN27016_c0_g1	*slc5a8*	5	365	73.0	0.002

Table 2.3. Top significantly enriched gene sets in small males NES: normalized enrichment score. BP: biological process. CC: cellular component. MF: molecular function.

Gene Set	Description	Type	NES	P-*adj*
GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	BP	2.79	0.00
GO:0007155	cell adhesion	BP	2.33	0.00
GO:0030054	cell junction	CC	2.31	0.00
GO:0005248	voltage-gated sodium channel activity	MF	2.30	0.00
GO:0004222	metalloendopeptidase activity	MF	2.32	0.00
GO:0035235	ionotropic glutamate receptor signaling pathway	BP	2.32	0.00
GO:0005234	extracellularly glutamate-gated ion channel activity	MF	2.27	0.00
GO:0005887	integral component of plasma membrane	CC	2.40	0.00
GO:0060078	regulation of postsynaptic membrane potential	BP	2.29	0.00
GO:0003777	microtubule motor activity	MF	2.56	0.00
GO:0007018	microtubule-based movement	BP	2.41	0.00
GO:0005328	neurotransmitter: sodium symporter activity	MF	2.37	0.00
GO:0006836	neurotransmitter transport	BP	2.33	0.00
GO:0018108	peptidyl-tyrosine phosphorylation	BP	2.29	0.00
GO:0003333	amino acid transmembrane transport	BP	2.28	0.00
GO:0015171	amino acid transmembrane transporter activity	MF	2.25	0.00
GO:0034765	regulation of ion transmembrane transport	BP	2.34	0.00
GO:0034220	ion transmembrane transport	BP	2.31	0.00
GO:0035725	sodium ion transmembrane transport	BP	2.27	0.00
GO:0048013	ephrin receptor signaling pathway	BP	2.27	0.00

Table 2.4. Eleven significantly enriched gene sets in large males NES: normalized enrichment score. BP: biological process. CC: cellular component. MF: molecular function.

Gene Set	Description	Type	NES	P-*adj*
GO:0003735	structural constituent of ribosome	MF	2.59	0.000
GO:0006412	translation	BP	2.49	0.000
GO:0005840	ribosome	CC	2.29	0.000
GO:0022625	cytosolic large ribosomal subunit	CC	2.27	0.000
GO:0022627	cytosolic small ribosomal subunit	CC	2.20	0.000
GO:0042613	MHC class II protein complex	CC	2.03	0.004
GO:0030240	skeletal muscle thin filament assembly	BP	1.94	0.011
GO:0005861	troponin complex	CC	1.84	0.031
GO:0005839	proteasome core complex	CC	1.80	0.040
GO:0006955	immune response	BP	1.80	0.038
GO:0002376	immune system process	BP	1.78	0.039

Details of differential gene expression and gene set enrichment between fixed mating strategies of small and large males

For the comparison between small and large males, most gene sets and DE genes could be binned into four categories that provide a framework for more detailed examination of gene expression differences between fixed mating strategies.

Ion channels and transport

Small males showed significant enrichment of gene sets related to ion transport (GO:0034220; GO:0035725), regulation of cellular cation transport (GO:0034765), ligand-gated ion channel function (GO:0005248) and the ionotropic glutamate signaling pathway (GO:0035235; GO:0005234; Table 2.3). Also, five of the top 20 up-regulated transcripts in small males were related to ion transport and the ionotropic glutamate signaling pathway.

Four up-regulated ion transport genes were members of the SoLute Carrier (SLC) family; their encoded proteins are located in cellular and subcellular membranes. The electrogenic sodium bicarbonate cotransporter-1 (*slc4a4*; 2.10 FC) plays a key role in mediating sodium/chloride-dependent transport of basic anions for fluid and electrolyte balance. It appears to be involved in fluid absorption from the cerebrospinal fluid to the brain parenchyma (Sussman *et al.* 2009). Two DE SLC genes (5.16 and 3.67 FC, respectively) were homologs of the sodium and chloride dependent glycine transporter *slc6a9* (Cui *et al.* 2005). *Slc6a9* appears to be important for optimizing muscle movement and neural signaling. Its missense mutation has been identified as the cause of the *sho* behavioral phenotype in zebrafish, i.e., abnormally persistent coupling between muscle

fibers and defective signaling in the CNS (Cui *et al.* 2005). The final DE SLC, an asc-type amino acid transporter (*slc7a10*; 3.36 LFC; Ehmsen *et al.* 2016), has not been experimentally characterized in fish. However, in mice, it plays an essential role in glycinergic inhibition in the central nervous system, which is essential for coordinated muscle movement (Ehmsen *et al.* 2016). Glutamate receptor 3 (*mglur3*, 2.10 FC) is also not well characterized in fish; in mammals, it inhibits adenylyl cyclase activity and various Ca^{2+} channels (Pin, Galvez & Prézeau 2003). Experiments with knockout *mGlu3R* gene mice highlight its involvement in synaptic plasticity (Pin, Galvez & Prézeau 2003).

Cell junction and signaling

Small males also showed significant enrichment of gene sets and DE genes related to cell adhesion (GO:0007156, GO:0007155, GO:0030054, GO:0048013), membrane proteins and membrane potential (GO:0005887, GO:0060078) and neurotransmission (GO:0005328, GO:0006836).

The pleckstrin homology domain-containing family A member 7 (*plekha7*; 2.99 FC) encodes a protein localized to the adherens junctions between vertebrate epithelial cells, including the blood cerebrospinal fluid barrier (Citi *et al.* 2012). *Plekha7* comprises the protein complex that links E-cadherin to the microtubule cytoskeleton at adherens junctions and is essential for structural intercellular connections (Citi *et al.* 2012). Another extracellular protein, *adamts20* (3.04 FC), plays a role in the turnover of extracellular matrix proteins in multiple tissues, including brain junctions (Tortorella *et al.* 2009).

Two DE genes were important regulators of synapses. Turtle homolog B (*tutlB*; 1.91 FC) codes for a vertebrate membrane adhesion molecule that regulates excitatory synaptic maturation in hippocampal neurons (Hansen & Walmod 2013). RIMS-binding 2 (*rimbp2*; 1.98 FC) also regulates synaptic transmission by serving as an adaptor and linking calcium channels with the synaptic vesicle release machinery (Wang *et al.* 2000).

Immunity

Large males showed significant enrichment of gene sets associated with immunity (GO:0042613 GO:0006955 GO:0002376), and four of the top 20 downregulated genes were also involved in immune response (Table 2.2). Two of the immune response genes, LRR and PYD domains-containing 12 (*nlrp12*; 4.06 FC) and growth arrest and DNA damage-inducible gene (*gadd45*; 3.63 FC), produce proteins involved in intracellular signaling and pathogen recognition (Fang *et al.* 2018; Laing *et al.* 2008). The remaining two, polymeric immunoglobin receptor (*pigr;* 56.1 FC) and V-set domain containing T cell activation inhibitor 1 (*vtcn1*; 41.0 FC), are extracellular membrane proteins involved in surface recognition of pathogens (Carmona *et al.* 2017; Rombout *et al.* 2008).

Ribosomes and protein translation

Almost 50% of the enriched gene sets in large males were related to translation and to structural constituents of large and small ribosomal subunits (Table 2.4; GO:0003735, GO:0005840,GO:0022625,GO:0022627; GO:0006412). One of the top 20 upregulated genes in large males, the pre-rRNA-processing TSR1 homolog (*tsr1*; 4.18 FC), was also associated with ribosomal construction and translation (Strunk *et al.* 2011).

TSR1 is a GTPase like protein required for 40S synthesis of the small ribosomal unit (Lebaron *et al.* 2012).

Overall differential gene expression and gene set enrichment results among social ontogeny treatments in intermediate males with more plastic mating strategies

To investigate differential gene expression due to social ontogeny treatment among more behaviorally plastic intermediate males, we examined transcriptional differences between pairwise comparisons of 15 intermediate males reared in the three social ontogeny treatments (Table 2.5).

Table 2.5. Number of significantly DE and annotated DE genes (protein coding and RNA coding) for intermediate males in pairwise social ontogeny treatment comparisons. Social ontogeny treatment, females: N = 4 males; large: N = 6 males; small: N = 5 males.

Comparison	DE genes	Annotated DE genes
Large vs. small	78	59
Females vs. small	326	150
Females vs. large	3	0

Fifty-nine protein coding genes were DE in intermediate males reared in the small male vs. large male social ontogeny treatments (FDR=0.05). The majority of these genes (49) were up-regulated in intermediate males reared in the large male social ontogeny treatment. All but one of these DE genes in the large male vs. small male social ontogeny comparison showed modest log fold changes (<2 LFC). Thirty-nine gene sets were significantly enriched when comparing intermediate males reared in the large and small

male social treatments (FDR=0.05). The majority (38 of 39) were enriched in the large male social ontogeny treatment group.

One hundred and fifty protein coding genes were differentially expressed in intermediate males reared in the females vs. small male social ontogeny treatments (FDR=0.05). More than half of these genes (82) were upregulated in the small male social treatment. All DE genes between intermediate males reared in the females and small male social treatment showed modest log fold changes (<2 LFC) and higher p-values than the other comparisons in this study. One hundred and two gene sets were significantly enriched when comparing intermediate males from the small male and females social ontogeny treatments (FDR=0.05). The majority (112 of 116) of the gene sets were enriched in males reared in the females social group.

Only three genes were DE in intermediate males reared in the large male vs. females social ontogeny treatments (Table 2.5; FDR=0.05). Furthermore, none of the DE genes were protein coding, suggesting that there is not a significant difference in protein coding gene expression for intermediate males when reared in the presence of large adult males or adult females. GSEA analysis of the large vs. females social treatments comparison did find twenty-seven gene sets that were enriched in intermediate males reared in the females social ontogeny treatment.

The small male social ontogeny treatment appears to have a stronger influence on gene expression patterns than the other two social ontogeny treatments. When comparing DE for intermediate males reared in the large vs. small male social groups and the females vs. small male social group, 58 genes (14 annotated) were consistently DE

(Table 2.6). Recall, few genes (N = 3) were DE in intermediate males reared in large male vs. females social ontogeny treatments. We also found that intermediate males reared in the small male social ontogeny treatment showed the most consistent differential expression of genes and hierarchical clustering patterns (seen above the heatmap in Figure 2.3) when compared to the other social ontogeny treatments (Figure 2.3). these comparisons intermediate males reared in the small social ontogeny treatment expressed two (annotated) genes at higher levels and twelve at significantly lower levels (Table 2.6). We also found 34 gene sets that were significantly depleted in males reared in the small male social ontogeny treatment regardless of comparison (Table 2.7).

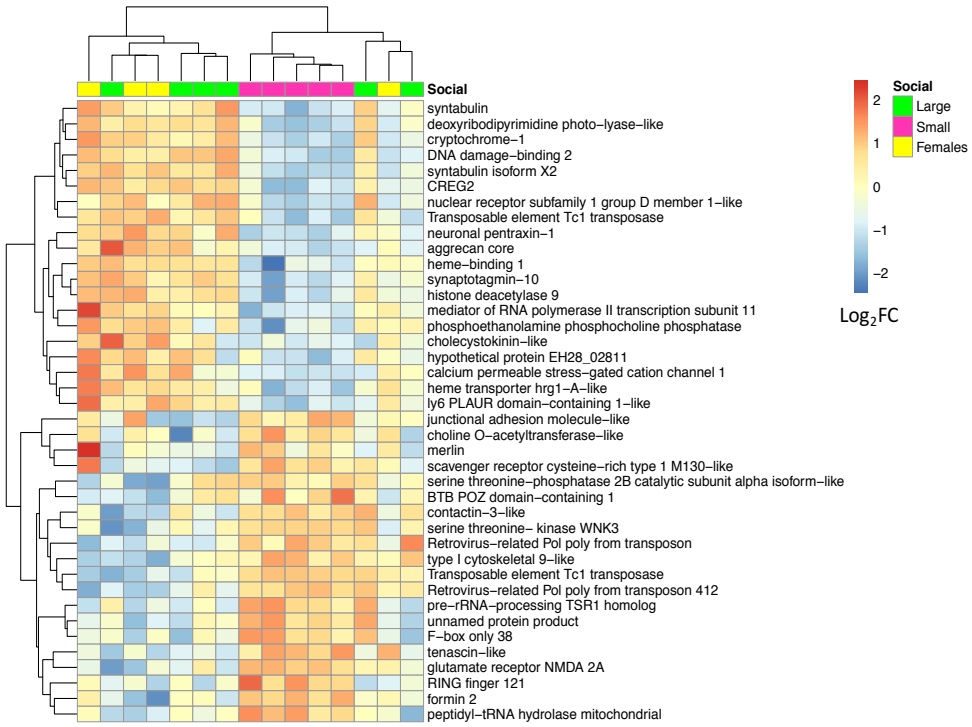

Fig. 2.3 Heatmap of Log2FC estimates and sample clustering for the top 40 DE genes sorted by p-value among social ontogeny treatments. Similarity between individuals with hierarchical clustering can be seen above the heatmap. Orange and blue indicate up-and down-regulation, respectively. Log2FC: Log2FoldChange.

Table 2.6. Consistently significantly DE genes in the small social ontogeny treatment across all comparisons.

		Normalized counts		
Sequence ID	Gene	Small	Large	Females
TRINITY_DN32402_c1_g3	*phrb*	262	516	464
TRINITY_DN29947_c4_g1	*syt10*	347	588	531
TRINITY_DN28964_c3_g1	*parl*	136	217	214
TRINITY_DN36108_c3_g1	*creg2*	181	347	318
TRINITY_DN30384_c2_g1	*hdac9*	714	1095	1060
TRINITY_DN34829_c1_g1	*lonrf1*	872	1424	1246
TRINITY_DN33509_c2_g1	*hebp1*	142	259	259
TRINITY_DN31034_c3_g4	*nell2*	565	763	768
TRINITY_DN33185_c6_g3	*nptx1*	2229	3331	3421
TRINITY_DN34161_c3_g5	*hnrnpc1*	1017	1297	1387
TRINITY_DN28885_c1_g2	*ankd63*	247	458	474
TRINITY_DN27747_c5_g1	*ciart*	531	850	870
TRINITY_DN36391_c6_g2	*tc1*	85	193	205
TRINITY_DN31807_c3_g3	unknown	1	6	43

Table 2.7. Consistently DE gene sets in the small social ontogeny treatment across all comparisons. NES: normalized enrichment score. BP: biological process. CC: cellular component. MF: molecular function.

Gene Set	Description	Type
GO:0006412	translation	BP
GO:0002181	cytoplasmic translation	BP
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	BP
GO:1902600	proton transmembrane transport	BP
GO:0007218	neuropeptide signaling pathway	BP
GO:0006364	rRNA processing	BP
GO:0042254	ribosome biogenesis	BP
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	BP
GO:0001731	formation of translation preinitiation complex	BP
GO:0006446	regulation of translational initiation	BP
GO:0043009	chordate embryonic development	BP
GO:0048666	neuron development	BP
GO:0006413	translational initiation	BP
GO:0032922	circadian regulation of gene expression	BP
GO:0038095	Fc-epsilon receptor signaling pathway	BP
GO:0006414	translational elongation	BP
GO:0002755	MyD88-dependent toll-like receptor signaling pathway	BP
GO:0019229	regulation of vasoconstriction	BP
GO:0005840	ribosome	CC
GO:0022625	cytosolic large ribosomal subunit	CC
GO:0022627	cytosolic small ribosomal subunit	CC
GO:0005730	nucleolus	CC
GO:0045095	keratin filament	CC
GO:0071011	precatalytic spliceosome	CC
GO:0005739	mitochondrion	CC
GO:0046540	U4/U6 x U5 tri-snRNP complex	CC

Details of differential gene expression and gene set enrichment among social ontogeny treatments in intermediate males with more plastic mating strategies

Patterns of DE for the 15 intermediate males could be binned into three categories that provide a framework for more detailed examination of gene expression differences among intermediate males that experienced different social group rearing conditions during ontogeny to sexual maturity.

Translation and Transcription

Interestingly, all of the gene sets related to ribosomes and translation that were enriched in large males (Table 2.4), were depleted in intermediate males reared in the small male social ontogeny treatment (GO:0003735, GO:0005840, GO:0022625, GO:0022627, GO:0006412) when contrasted with those from intermediate males reared with either females or the large male social group (Table 2.7).

Circadian rhythm and light responsiveness

Three of the 12 top downregulated protein coding genes among intermediate males reared in the small male social ontogeny treatment for both comparisons (vs. females and large male groups) were associated with circadian rhythm and UV damage repair (Table 2.6). This was confirmed by the significantly depleted gene set (GO:0032922) that is associated with the circadian regulation of gene expression (Table 2.7). The circadian-associated transcriptional repressor (*ciart*) forms a negative regulatory component of the circadian clock (Takahashi 2017). Deoxyribodipyrimidine photo-lyase (*phrb*) catalyzes and repairs cyclobutane pyrimidine dimers, which are formed between adjacent bases on the same DNA strand upon exposure to ultraviolet

radiation (Yasuhira & Yasui 1992; Hsu *et al.* 2001). The LON peptidase N-terminal domain and RING finger 1 (*lonrf1*) has an unknown function but has been found across fish species to be highly upregulated in response to light (Weger et. al 2011; Okano *et al.* 2017).

Synaptic plasticity

Interestingly, there were three genes downregulated in intermediate males reared in the small male social ontogeny treatment that have been associated with synaptic plasticity (*ntpx1, syt10 and tsc1*). *Nptx1* has been found in mammals to be involved in regulating uptake of synaptic material during synapse remodeling (Appelbaum *et al.* 2010). *Syt10* is typically involved in the regulated exocytosis of vesicles and can act as a calcium sensor in vesicular fusion processes (Woitecki *et al.* 2016). Intermediate males reared in the small social ontogeny treatment also showed downregulation of the *tc1* gene, the same transposase that was upregulated in small males and has been found to have a strong correlation with groups of genes involved in neural signaling (Krasnov *et al.* 2005).

DISCUSSION

We investigated brain gene expression patterns of male sailfin mollies at the developmental point of sexual maturity, which exhibit both 'fixed' and 'plastic' patterns of mating behavior strategies based upon body size (SL) differences.

Gene expression profiles between fixed mating strategies of small and large males

In agreement with a prior gene expression study in sailfin mollies (Fraser *et al.* 2014), we found approximately 2000 DE protein coding genes between the two fixed mating behavior extremes of the male size distribution, 'small' and 'large' males. However, the neurogenomic response was not as broad in our results as those previously described (~15%) between 'courter' and 'sneaker' males in fishes (Aubin-Horth *et al.* 2005a; Fraser *et al.* 2014). We found approximately 2% of all protein coding transcripts were DE between small and large males. This smaller difference in DE of genes between small and large males may be due to using the sailfin molly transcriptome we constructed in this study for mapping DE genes. The previous gene expression comparison between small and intermediate males relied on the well annotated, though more phylogenetically distantly related, guppy (*P. reticulata*) genome (Fraser *et al.* 2014). Also, no large males were included in the Fraser et al. (2014) study and intermediate males with more plastic mating strategies may have broader expression differences than between males of alternative fixed mating strategies.

Our findings indicated that genes related to cognition (learning and memory) were up-regulated in small males. Previous studies in fish species with alternative mating strategies indicate that sneaker males have increased expression of genes related to

neurotransmission and learning (Aubin-Horth *et al.* 2005; Fraser *et al.* 2014; Schunter *et al.* 2014). Interestingly, concordant with findings in both sailfin mollies and bluegill sunfish (*Lepomis macrochirus*), the ionotropic glutamate signaling pathway and ionotropic glutamate receptor activity GO terms were enriched in small males (Fraser *et al.* 2014; Partridge *et al.* 2016). Glutamate neurotransmission participates in neuronal plasticity and ultimately underlies the fundamental processes of information storage in the brain including spatial working memory (Foulkes & Sassone-Corsi 1992; Partridge *et al.* 2016). Small sneaker males across poeciliid taxa might benefit from increased spatial working memory as they position themselves for sneaking copulations with the female. Although, it is important to note that the large males also had significant upregulation of a NMDA receptor (*grin1*) that has also been found to be associated with spatial working memory in fish (Gómez *et al.* 2006).

Small males also showed GO term enrichment and upregulation of genes related to cell adhesion. In vertebrate brains, forming new synaptic contacts involves structural alterations and regulation of adhesion between the pre-and postsynaptic neuron (Lamprecht & LeDoux 2004). Adhesion molecules and their regulators not only influence extracellular connectively but also can induce intracellular changes in cytoskeleton morphology including dendritic spine morphogenesis (Lamprecht & LeDoux 2004). Dendritic spines provide anatomical substrate for memory storage in the synapse (Lamprecht & LeDoux 2004). Therefore, adhesion molecules contribute to the morphological alteration and stabilization of connectivity between neurons, a process that is hypothesized to underlie memory consolidation and learning (Schmidt 1994). The

upregulation of genes and gene sets associated with cognition and cell adhesion suggest that small males might have different cognitive demands imposed by the sneaker male mating strategy.

In contrast to small males, large males had increased enrichment of translation related gene sets (Table 2.5), suggesting that large males are prioritizing wholesale induction of translational machinery and *de novo* protein synthesis. Furthermore, translation related GO term enrichment is usually associated with increased growth and metabolic processes (Renn *et al.* 2008).

Large males also showed GO term enrichment and large fold change upregulation of multiple genes associated with immune function. There does appear to be a link between impaired immune function and social status in males across many teleost taxa (Yada & Nakanishi 2002; Liljedal & Folstad 2003; Tort 2011). Subordinate males are typically found to be more susceptible to bacterial and viral infections than dominant individuals (Peters *et al.*, 1988; Pottinger and Pickering, 1992), reflecting a poorer condition of subordinate fish. However, this upregulation found in our study might simply be due to large males taking a longer time to reach sexual maturity, and therefore, experiencing a longer period of time to encounter immune pathogens and build immunological resistance.

Gene expression profiles among social ontogeny treatments in intermediate males with plastic mating strategies

Among intermediate males we did find an effect of social ontogeny treatment on gene expression profiles, though the effect appears moderate both in terms of numbers of

genes DE and overall fold changes. When examining the fixed strategies results above and previous findings of large gene expression differences between small and intermediate males (Fraser *et al.* 2014), these moderate changes suggest that fixed mating strategies have greater transcriptional differences between them than those found among intermediate males with more plastic mating phenotypes.

Overall, we found the small male social ontogeny treatment to have the most directional effect on both male size at maturity and differential gene expression patterns in intermediate males. Though we did not find a significant influence of social ontogeny on size at maturity, males maturing in the small social ontogeny treatment displayed the least amount of variance in SL, with most maturing at intermediate size; indeed, no males reared in the small male social ontogeny treatment matured at small male size (Figure 2.1). We also found that when examining clustering analysis, the intermediate males reared in the small male social group showed the tightest clustering of DE genes (Figure 2.3) and had the least transcriptional variance when compared to those reared in the large male and females social ontogeny treatments. The influence of being raised in a small male social environment on increasing male size at maturity and differential gene expression could in part, explain the switch from a small male-dominated social structure early in the breeding season to one of a higher frequency of intermediate and large males in late summer and early fall that has been repeatedly observed in the Steve's Ditch population (Travis, Ptacek and Lange pers. obs.).

Males reared in the small male social ontogeny treatment showed consistent downregulation of genes and gene sets associated with translation, circadian rhythm and

synaptic plasticity. Depletion of gene sets associated with translation and protein synthesis suggests that intermediate males reared with small males may use social cues from small male group members to suppress translational machinery typically associated with growth (Renn *et al*. 2008).

Our finding that intermediate males reared in the small male social ontogeny treatment had a significant downregulation of genes and gene sets related to circadian rhythm compared with males reared in the other two social ontogeny treatments is unexpected. These differences were not due to light or feeding cycle differences among social ontogeny treatments since all experimental fish had the same diel light patterns and feeding schedule. Experimental fish were also sacrificed at the same time of day to avoid gene expression patterns attributed to the circadian rhythm instead of experimental testing. In salmonid fishes, socially subordinate individuals avoid competition with dominant fish by adjusting daily feeding and activity cycles (Larson *et al.* 2004). These modifications limit interactions with the larger dominant individuals. In *P. latipinna*, large males harass and chase smaller males (Farr, Travis & Trexler 1986). Thus, circadian rhythm genes and gene sets might be worth exploring in future studies of the influence of social environment on life history and mating behaviors in sailfin mollies.

In the present study, we only analyzed differential gene expression patterns in male *P. latipinna* at the time of maturity. Future research will be carried out to obtain a comprehensive understanding of the molecular mechanisms underlying timing and size at maturity in male sailfin mollies, including epigenetic regulation, alternative gene splicing, and post-transcriptional modifications.

CONCLUSION

The largest differences in gene expression levels were observed when comparing fixed alternative mating strategies (small sneaker males vs. large courting males), suggesting that differences in gene expression are more related to male reproductive strategy than to social rearing environment during ontogeny for the extreme size classes of male sailfin mollies. Consistent with other studies, our work demonstrates that sneaker males have greater expression of genes involved in neural function relative to courting males, particularly in relation to spatial working memory. Our results from patterns of DE genes among intermediate males reared in different social contexts with males of different sizes suggests that social environment, particularly association with small males, may contribute to increased mating behavior plasticity. The seasonal variation in the size distribution of mature males in the social environment of natural populations of mollies, often with a high frequency of small males in early spring followed by more intermediate and large males later in the breeding season, could significantly impact gene expression patterns in intermediate males leading to the increased plasticity of mating behaviors observed in this size class of males. Such plasticity in mating behavior repertoires in response to social cues may, in part, explain the maintenance of male size polymorphism and alternative mating strategies in sailfin mollies.

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